

Segregation of Bovine Viral Diarrhea Virus into Genotypes¹

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Isolates of bovine viral diarrhea virus (BVDV) were segregated into two groups based on comparison of sequences from the 5' untranslated region (UTR) of the viral genome. Phylogenetic analysis suggested that these groups, termed BVDV I and BVDV II, are as different from each other as reference BVDV (BVDV-NADL, BVDV-SD-1, BVDV-Osloss) are from hog cholera virus. Polymerase chain reaction (PCR) tests, based on the 5' untranslated region and the genomic region coding for the p125 polypeptide, were designed to differentiate between BVDV I and BVDV II. Using these tests, 76 of 140 isolates of BVDV were identified as BVDV II. Antigenic and pathologic differences were noted between BVDV I and BVDV II viruses. Among BVDV I were viruses commonly used in vaccine production, diagnostic tests, and research. BVDV II was isolated predominantly from fetal bovine sera, persistently infected calves born to dams vaccinated against BVDV, and cattle that had died from an acute form of BVDV termed hemorrhagic syndrome.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a ubiquitous pathogen of cattle. Along with hog cholera virus (HCV) of swine and border disease virus (BDV) of sheep, BVDV is classified as a member virus of the genus Pestivirus in the family Flaviviridae (Wengler, 1991). Pestiviruses have a single strand of positive-sense RNA that is approximately 12.5 kb in length and contains one large open reading frame (ORF) (Collett *et al.*, 1988). The large ORF is preceded by a 361- to 386-base untranslated region (UTR) (Collett *et al.*, 1988; Meyer *et al.*, 1989; Moormann *et al.*, 1990; Deng and Brock, 1992). Because the 5' UTR is highly conserved among pestivirus species, it has been proposed that sequences from this region can be used to differentiate among the member viruses (Boye *et al.*, 1991; Berry *et al.*, 1992; Qi *et al.*, 1992; De Moerloose *et al.*, 1993; Ridpath *et al.*, 1993). Another member of the family Flaviviridae, hepatitis C virus, has a genomic organization that is similar to pestiviruses. There is sequence similarity between pestiviruses and hepatitis C virus in the 5' UTR (Choo *et al.*, 1991). Isolates of hepatitis C virus have been divided into genotypes based on comparison of sequences in the 5' UTR (Bukh *et al.*, 1992; Cha *et al.*, 1992; Simmonds *et al.*, 1993).

Serologic subgroups of BVDV are not recognized, but

several reports document significant genomic and antigenic heterogeneity among BVDV. Nucleic acid hybridization analysis of BVDV isolates has detected differences of 40% among viral isolates in select regions of the genome (Ridpath and Bolin, 1991b). Antigenic variation among BVDV isolates has been detected using polyclonal and monoclonal antibodies (Hafez and Liess, 1972; Itoh *et al.*, 1984; Howard *et al.*, 1987; Bolin, 1988; Bolin *et al.*, 1988, 1991a; Shimizu *et al.*, 1989; Ridpath and Bolin, 1991a). This heterogeneity among BVDV isolates is likely responsible for the failure of both serologic- and genomic-based diagnostic reagents to detect all isolates of BVDV (Heuschele, 1975; Boye *et al.*, 1991; Hertig *et al.*, 1991; Kwang *et al.*, 1991; Lewis *et al.*, 1991; Ward and Misra, 1991). Further, isolation of BVD strains that escape neutralization by vaccine-induced antibody suggests that antigenic variation may contribute to vaccine failure (Bolin *et al.*, 1991a).

In this study, we characterized isolates of BVDV using genomic criteria. Phylogenetic analysis, based on sequences from the 5' UTR, segregated BVDV isolates into distinct subgroups. Antigenic and pathologic differences, noted between these subgroups, may have practical significance *in vivo*.

MATERIALS AND METHODS

Materials

Restriction enzymes and modifying enzymes were obtained from Boehringer Mannheim (Indianapolis, IN) and BRL (Gaithersburg, MD). Custom polymerase chain reaction (PCR) primers were synthesized by IDT, Inc. (Coralville, IA). Vector systems were purchased from In-

¹ Product names are necessarily included to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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vitrogen, Corp. (San Diego, CA). Automated sequencing kits were obtained from Applied Biosystems (Foster City, CA) and E. I. Du Pont De Nemours & Co. (Boston, MA). Reagents for agarose and polyacrylamide gel electrophoresis (PAGE) and phenol/chloroform solutions were purchased from Amresco (Solon, OH). Minicolumns and microconcentrators used to partially purify sequencing templates were obtained from Qiagen, Inc. (Catsworth, CA) and Amicon, Inc. (Beverly, MA), respectively. Radioactive compounds were purchased from ICN (Costa Mesa, CA).

Viral isolates

The BVDV isolates ($n = 140$) analyzed here originated in either the United States or Canada and were isolated between 1960 and 1993. They represented laboratory reference strains, vaccine strains, and field strains isolated from diseased cattle or from fetal bovine serum. All BVDV were biologically cloned by serial passage at limiting dilution. Bovine turbinate (BT) cells were used to propagate BVDV. The fetal bovine serum used to supplement cell culture medium was tested and found to be free of adventitious BVDV and antibodies against BVDV (Bolin *et al.*, 1991b).

Genomic comparison of BVDV

For genomic comparison of BVDV isolates, portions of the viral genome were amplified by PCR and sequenced. PCR primers were selected based on conservation between published sequences of BVDV and HCV (Collett *et al.*, 1988; Meyer *et al.*, 1989; Moormann *et al.*, 1990; Deng and Brock, 1992). Primers were designed using the Primer 2 program (Scientific and Educational Software, State Line, PA). Sequence comparison and phylogenetic analysis were performed using the Align Plus software program (Scientific and Educational Software, State Line, PA) and the GeneWorks software package (Intelligenetics Inc., Mountain View, CA). To generate viral template, total RNA from BVDV-infected BT cells was prepared by acid guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski and Sacchi, 1993) as adapted for tissue culture cells (Kingston *et al.*, 1993). This procedure was modified by using 5/1 phenol/chloroform (pH 4.7) instead of water-saturated phenol. RNA was harvested from BT cells 14 to 24 hr after infection with cytopathic BVDV or 48 hr after infection with noncytopathic BVDV. For sequence analysis, a 2- μ g aliquot of RNA was heated to 90°C for 5 min. To this aliquot was added the RT/first-round PCR reaction mixture [2.5 units *Taq* DNA polymerase, 50 units MLV-reverse transcriptase, 200 units RNase inhibitor, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM each in dATP, dCTP, dGTP, and dTTP, 1.5 mM MgCl₂, 0.1% Triton-X 100, and 100 pM forward and reverse primers in a 100- μ l volume].

When applicable, second-round PCR reactions were

TABLE 1
PRIMER SEQUENCES AND PCR REACTION CONDITIONS

Primer set		Equivalent position in BVDV-NADL
A (First-round PCR primers)		
Forward	gta gtc gtc agt ggt tcg	188
Reverse	gcc atg tac agc aga gat	366
B (Second-round PCR primers)		
Forward	cga cac tcc att agt tga gg	191
Reverse	gtc cat aac gcc acg aat ag	296
C		
Forward	gca gtc aca ata gga gag	6902
Reverse	tgt aga gtg ctg tgt gag	7869
RT reaction		
1 cycle: 56°C, 1 hr		
94°C, 4 min		
PCR reaction (1st round)		
25 cycles: 94°C, 10 sec		
50°C (Primer set A)		
or		
45°C (Primer set C)		
72°C, 45 sec		
1 cycle: 72°C, 10 min		
PCR reaction (2nd round)		
25 cycles: 94°C, 10 sec		
50°C, 30 sec		
72°C, 30 sec		
1 cycle: 72°C, 10 min		

performed as follows. A 15- μ l aliquot of first-round amplicon was electrophoresed in 1.0% low-melting-point agarose gel and stained with ethidium bromide. A plug was taken from the amplicon band in the electrophoresis gel using a sterile cotton-plugged pipette tip. The plug was expelled into 5 μ l sterile distilled water and added to the second-round reaction mixture [2.5 units *Taq* DNA polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM each in dATP, dCTP, dGTP, and dTTP, 1.5 mM MgCl₂, 0.1% Triton-X 100, and 100 pM each of second-round PCR primers] to a final volume of 100 μ l. Reaction conditions and primer sequences are shown in Table 1. Identity of amplicons was confirmed by nucleic acid hybridization (Ridpath and Bolin, 1991a).

For the first 20 isolates sequenced, amplicons were cloned into a bacterial vector system and subsequently sequenced. At least three different plasmids were sequenced for each amplicon that was cloned into a bacterial vector. Three viral sequences derived in this manner were also derived by direct sequencing of the PCR amplicon. No differences were seen between sequences derived by these two methods. Subsequently, all viral sequences were determined by direct sequencing of amplicons, due to cost and time savings associated with this method. All direct sequencing reactions were run in duplicate. Before sequencing, amplicons and plasmids were partially purified by column chromatography or by centrifugation through microconcentrators. Sequences were determined by the Sanger method or by cycle se-

BVDV-NADL	188	GTAGTCGTCAGTGGTTTCGACGCC---TTGGAATAAAGGTCTCGAGATGCCACGTGGACGAGGGCATGCC	
BVDV-SD-1	188C..T.....C....	
890	A.TCCT..A.TCG.GGA.....T.....	
AZ Spl		..C.....A.TCCA.CA.CTG.GGA.....T.....	
Mad Spl		...G.....A.TCCA..A.TTG.GGA.....T.....	
Mn Fetus	A.TCCA..A.TCG.GGA.....T.....	
HCV-Alfort	170	AC.....A.....TG---AGCACTAGCCAC.....T.....	
HCV-Brascia	167	AC.....A.....TG---AGCAG.AGCCAC.....T.T.....	
		AAAGCACATCTTAACCTGAGCGGGGTCGCCAGGTAAAGCAGTTTAAACCGACTGTTACGAATACAGC	
	C.....A.CG.C.AAT.....	
		.CG.....CAC.....T..GTG...G.....CCA...--GTGGC..C.T.G.C....	
		.CG.....AT.....T..ATG...G.....GCCA...--GTGG..C.T..C....	
		.CG.....CAT.....T..ATG...G.....GC.A...--GTGGC...T.G.C....	
		.TG.....CAT.....T..ATG...G.....GC.A...--GTGGC...T.G.C....	
		..GA...C.....CTG.....TAG...G...T..CA.....--ATG..A.GG.GC...GA.	
		..GA...C.....TTAG...G...T.....C...ATG..A.GG..G...GA.	
		CTGATAGGGTGTGTCAGAGGCCCACTGTATTGCTACTA--AAAATCTCTGCTGTACATGCC	383
		383
	TA.....A..TG..ACTCC...G.....	
	TA.....AA.TG..A.TCC...G.....	
	TA.....A..TG..ACTCC...G.....	
	TA.....A..TG..ACTCC...G.....	
	AGCAG...G..TA.....	362
	A.TAG...G..TA.....	358

Fig. 1. Alignment of the 5' UTR from BVDV isolates associated with hemorrhagic syndrome (HS) with the 5' UTR from published pestivirus sequences. The region of the 5' UTR corresponding to BVDV-NADL base position 188 through 383 was amplified and sequenced from BVDV isolates associated with HS. These sequences were aligned with the equivalent published sequences from BVDV-NADL (Collett *et al.*, 1988), BVDV-SD-1 (Deng and Brock, 1992), HCV-Alfort (Meyer *et al.*, 1989), HCV-Brescia (Moormann *et al.*, 1990). An example of these comparisons, using 4 BVDV isolates (890, AZ spl, Mad spl, MN fetus), is shown. All sequences are represented in comparison to BVDV-NADL. Bases are shown for nonmatches to the BVDV-NADL sequence. The percentage homology to BVDV-NADL is as follows: 94% BVDV-SD-1, 77% 890, 77% AZ Spl, 77% Mad Spl, 77% Mn fetus, 73% HCV-Alfort, 75% HCV-Brescia.

quencing, as adapted for automated sequencing per the manufacturer's instruction. All sequences were confirmed by sequencing both strands.

Antigenic characterization of BVDV

Comparison of BVDV at the antigenic level was done using a monoclonal antibody (Mab) binding assay. The 29 Mabs used possessed viral neutralizing activity and reacted with BVD viral glycoprotein gp53 [also referred to as E2 (Thiel *et al.*, 1991) and E1 (van Rijn *et al.*, 1993)]. Mabs were prepared and characterized as described earlier (Bolin *et al.*, 1988). Mab binding was assessed by indirect immunoperoxidase staining of infected cell monolayers (Bolin *et al.*, 1991b). Radioimmunoprecipitation (RIP) profiles of BVDV isolates were generated, as described previously (Ridpath and Bolin, 1990), using hyperimmune bovine serum. As a control, uninfected ³⁵S-labeled cell lysates were also immunoprecipitated with the hyperimmune serum.

RESULTS

Characterization of BVDV associated with hemorrhagic syndrome

We initially examined BVDV isolated from outbreaks of hemorrhagic syndrome (HS). HS results from an acute, uncomplicated infection of cattle with BVDV and is characterized by leukopenia, fever, diarrhea, thrombocytopenia, hemorrhaging, and death (Corapi *et al.*, 1989, 1990; Rebhun *et al.*, 1989; Bolin and Ridpath, 1992). Upon

analysis it was apparent that genomic sequences from the 5' UTR (Fig. 1) of BVDV isolates associated with outbreaks of HS were clearly distinct from published sequences of BVDV (Collett *et al.*, 1988; Deng and Brock, 1992) or HCV (Meyer *et al.*, 1989; Moormann *et al.*, 1990). The aligned sequence identities in the 5' UTR, among BVDV isolates associated with outbreaks of HS, were 90% or greater. However, the aligned sequence identities between BVDV associated with HS and published sequences of BVDV or HCV were less than 70%.

Similar relationships were observed among predicted amino acid sequences from the region of the genome coding for the p125 viral polypeptide (Fig. 2). Once again, BVDV isolates associated with HS outbreaks were more similar to each other than they were to BVDV or HCV isolates previously characterized in the literature.

Phylogenetic analysis of BVDV

For phylogenetic analysis, a portion of 5' UTR, corresponding to BVDV-NADL base positions 188 to 383, was amplified and sequenced from 44 BVDV isolates. For this analysis we also included sequences from two HCV (Meyer *et al.*, 1989; Moormann *et al.*, 1990) and two BDV (Berry *et al.*, 1992). Phylogenetic analysis grouped these pestiviruses into three separate branches (Fig. 3). The sequence similarity among viruses on the same branch was 88% or greater. The sequence similarity between viruses from different branches was 70% or less. One branch (branch 2, Fig. 3) consisted of the two HCV isolates. The 44 BVDV isolates were segregated into two

BVDV-NADL	2172	AVTVGEQAQRRGRVGRVKPCGRYYRSQETATGSKDYHYDLLQAQRYGIEDGINVTKSFREMNYDWSLYEED
BVDV-SD-1	2082S.....
890	S.....
AZ Spl	S.....
Mad Spl	S.....S.....
Mn Fetus	PV.....I.....
HCV-Alfort	2082PV.....I.....
HCV-Brescia	2081PV.....I.....
		SLITITQLEILNLLISEDLPAAVKNIMARTDHPPIQLAYNSYEVQVPVLPFKIRNGEVTDTYENYSFLN
	M.....V.....H.N.I.....L.....K.....S.....TY..
	M.....V.....N.....K.....S.....TY..
	M.....V.....T.....N.I.....K.....S.....TY..
	M.....V.....N.I.....K.....S.....TY..
	M.....E.M.....T.....D.....T.....
	M.....E.M.....T.....K.....S.D.....T.....
		ARKLGEDVPVYIYATEDEDLAVDLLGLDWPDPGNQVQVETGKALKQVTGLSSAENALLVALFGYVGYQAL
	V.....G.....M.S.S.....R.....A.....T.....I.....T.....
	A.V.....M.....R.....T.....I.....T.....
	A.V.P.....M.....A.....R.....T.....I.....T.....
	A.V.....M.....R.....T.....I.....T.....
	D.....P.V.....E.....GT.A.R.....V.....T.....
	D.....P.V.....E.....GT.A.R.....V.....T.....
		SKRHVPMTIDYITIEDQRLDPTTHLQYAPNAIKTDGTETELKELASGDVEKIMCAISDYAAGGLEFVKSQ
	I.V.....L.H.....F.....R.....KDS.....V.....LD.YLD.LV.....SKQ.MK.I.V.....
	I.....L.H.....F.....R.....KDS.....V.....FD.YVD.LV.....SKQ.IK.I.V.....
	I.....L.H.....F.....R.....KDS.....V.....LD.YVD.LV.....SKQ.MK.I.V.....
	I.VV.....SV.....H.....E.K.....Q.....QRCVE.VTN.....RE.IQ.M.....
	I.VV.....S.....H.....E.K.....Q.....QRC.E.MTN.....RD.IQ.M.....
		AEKIKTAPLPFKENAEAAKGYVQKFIDSLIENKEEIIIRYGLWGHTALY
	2500V.....R.....L.....D.DV.....
	2410V.DSQST.....GLQTI.....E.D.....Q.....T.....A.....
	V.DSQST.....GLQTI.....E.D.....Q.....T.....
	VRDSQST.....GLHTI.....E.D.....Y.....Q.....T.....
	V.DSQAT.....GLHTI.....E.D.....Q.....A.....
	2410L.VRET.TY.....TMNTVAD.....K.....EA.TDS.....D.....K.....A.....
	2409L.V.ET.TY.....TMDTVAD.....K.....MEA.ADS.....D.....K.....

Fig. 2. Alignment of predicted amino acid sequences, from the region of the genome coding for the p125 viral polypeptide, derived from genomic sequences of BVDV isolates associated with hemorrhagic syndrome (HS) with the equivalent predicted amino acid sequences derived from published pestivirus sequences. A portion of the genomic region coding for p125, (corresponding to BVDV-NADL base positions 6902 through 7869) was amplified and sequenced from BVDV isolates associated with HS. The predicted amino acid sequence was generated from these genomic sequences and aligned with the equivalent predicted amino acid sequences generated from published sequences of BVDV-NADL (Collett *et al.*, 1988), BVDV-SD-1 (Deng and Brock, 1992), HCV-Alfort (Meyer *et al.*, 1989), and HCV-Brescia (Moormann *et al.*, 1990). An example of these comparisons, using four BVDV isolates (890, AZ spl, Mad spl, MN fetus), is shown. All sequences are represented in comparison to BVDV-NADL. Amino acids are shown for nonmatches to the BVDV-NADL sequence. The percentage homology to BVDV-NADL is as follows: 97% BVDV-SD-1, 81% 890, 84% AZ spl, 82% Mad spl, 83% Mn fetus, 80% HCV-Alfort, 80% HCV-Brescia.

different branches. Of the two branches containing BVDV isolates, one branch (branch 1, Fig. 3) contained many of the BVDV isolates commonly used in BVDV research (e.g., BVDV-NADL, BVDV-Singer, BVDV-C24V, BVDV-NY-1). The other branch (branch 3, Fig. 3) contained all the viruses that were associated with HS. In addition, this branch also contained BVDV isolates from persistently infected animals born to dams that had been vaccinated against BVDV, BVDV isolates from fetal bovine serum, and the two BDV isolates. For the remainder of this report we shall refer to the BVDV isolates from the branch containing BVDV-NADL and related viruses (branch 1, Fig. 3) as BVDV I. The viruses from the branch containing BVDV associated with HS and related viruses (branch 3, Fig. 3) will be referred to as BVDV II.

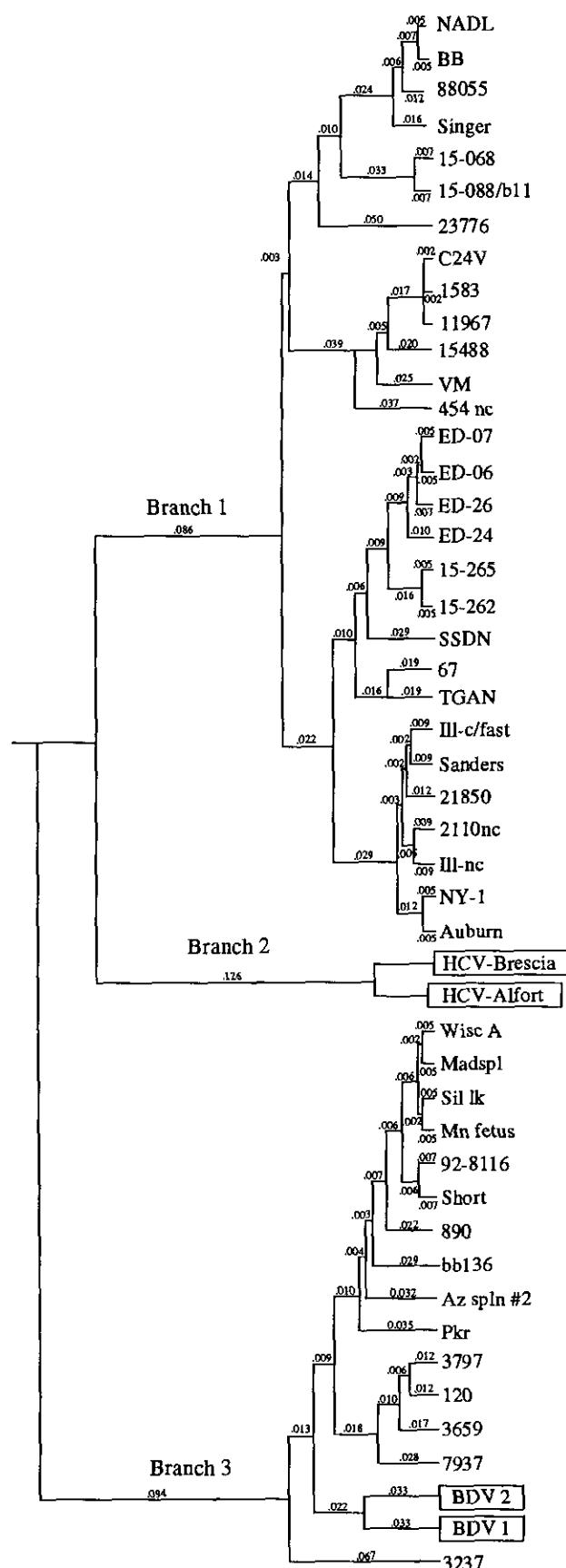
As stated above, BVDV II isolates and BDV isolates were segregated to the same branch on the phylogenetic tree (Fig. 3). While similarities were found in the 5' UTR, BDV isolates were distinct from BVDV II by Mab binding, PCR amplification, and growth characteristics in ovine- and bovine-derived cells (Ridpath *et al.*, 1992). Further,

we have inoculated sheep with BVDV associated with outbreaks of HS and have not observed any clinical signs of disease. Similarly, calves inoculated with BDV did not show signs of disease.

Characterization of BVDV II

The Mab binding patterns were determined for the viral isolates included in the phylogenetic analysis. The Mab binding patterns were highly similar among BVDV II isolates (Table 2), while the Mab binding patterns of the BVDV I isolates were more heterogeneous. It should be noted, however, that the Mabs used in this study were prepared against BVDV I isolates. The Mabs that reacted with BVDV II isolates likely bind epitopes that are conserved between BVDV I and II isolates. The consistent Mab binding pattern observed among BVDV II isolates may be more a function of the high conservation of a few epitopes between BVDV I and BVDV II isolates than a result of marked antigenic similarity among BVDV II isolates.

We observed differences in biotype and viral polypep-



tides among BVDV II isolates that suggested that these viruses are heterogeneous. A marked variation was seen in the size of the gp53 polypeptide among BVDV II isolates (Fig. 4). Variation in the size of the gp53 polypeptide has also been observed among BVDV I isolates (Donis and Dubovi, 1987; Ridpath and Bolin, 1990).

Similar to BVDV I isolates (Lee and Gillespie, 1957; Gillespie *et al.*, 1960, 1962), we identified cytopathic and noncytopathic BVDV II isolates based on their ability to induce cytopathic effect in cultured cells. Like cytopathic BVDV I isolates (Donis and Dubovi, 1987), cytopathic BVDV II isolates produce a polypeptide in the 80-kDa size range (Fig. 4a) not observed with the noncytopathic BVDV II isolates (Fig. 4b). The band seen in the 80-kDa range in RIPs of cytopathic BVDV II had a slightly slower mobility than the band observed in RIPs of cytopathic BVDV I (Fig. 4a).

Differentiation of BVDV II isolates from BVDV I isolates

We designed two PCR tests, based on conserved regions in the BVDV genome, to differentiate between BVDV I and BVDV II isolates. The first test was a nested PCR reaction that amplified sequences from the 5' UTR. The first-round primers (set A, Table 1) were used in an earlier study to differentiate BVDV from other pestiviruses. These primers amplified sequences from all BVDV isolates tested (Ridpath *et al.*, 1993). The second-round primers (set B, Table 1) selectively amplified BVDV II but not BVDV I sequences. A second PCR test was based on amplification of sequences from the region of the genome coding for the p125 polypeptide. Primer set C (Table 1) selectively amplified sequences within this region from BVDV II isolates but not BVDV I isolates. We analyzed 140 BVDV II isolates using these two PCR tests. Of these viruses, 76 were identified as BVDV II isolates (Table 3).

We also determined the Mab binding patterns for the 140 isolates listed in Table 3. All BVDV identified as BVDV II isolates by PCR ($n = 76$) had Mab binding patterns that were highly similar to those isolates segregated into branch 3 in the phylogenetic tree shown in Fig. 3. Viruses ($n = 64$) identified as BVDV I had variable Mab binding patterns. The viruses identified as BVDV II were isolated predominantly from persistently infected animals born to

Fig. 3. Phylogenetic analysis of BVDV. A portion of the 5' UTR, corresponding to BVDV-NADL base positions 188 through 383, was amplified and sequenced from 44 isolates of BVDV. Phylogenetic analysis of these 44 sequences was then done using an unweighted pair group method. The length of the horizontal lines connecting one sequence to another is proportional to the estimated genetic distance between the sequences. The phylogenetic tree was constructed by doing a pairwise position by position comparison of all sequences, followed by clustering the sequences by similarity. Scores were calculated by dividing the number of mismatches by the length of the shorted sequence.

TABLE 2

[illegible]

Note. Mabs listed on left. BVDV isolates listed across top. A dark square (■) indicates binding.

dams that had been vaccinated against BVDV, outbreaks of hemorrhagic syndrome, or from fetal bovine serum.

DISCUSSION

Our results indicate BVDV can be segregated into two subgroups—termed BVDV I and BVDV II. Phylogenetic analysis, based on comparison of sequences from the 5' UTR, suggests that BVDV II are as distinct from BVDV I as they are from HCV. The differences found between BVDV I and BVDV II in the 5' UTR are greater than those observed among the putative genotypes of hepatitis C virus (Bukh *et al.*, 1992; Cha *et al.*, 1992; Simmonds *et al.*, 1993). Thus by criteria used to subgroup hepatitis C virus, BVDV I and BVDV II represent different genotypes of BVDV.

Antigenic dissimilarity between BVDV I and BVDV II is suggested by the failure of most of the Mabs, prepared against the gp53 polypeptide of several BVDV I, to bind BVDV II. Several of the viruses identified as BVDV II were isolated from persistently infected animals born to dams that had been vaccinated against BVDV. We reported the isolation and partial characterization of one of these

isolates in an earlier study (Bolin *et al.*, 1991a). This virus, characterized in this report as a BVDV II, escaped neutralization by antibodies raised by vaccination with a virus identified as a BVDV I. The failure of antibodies, raised against a BVDV I virus, to protect against infection with a BVDV II has practical significance in vaccine development and control of BVDV. Further studies are now underway in our laboratory to examine cross-protection between BVDV I and Type II viruses and evaluate the efficacy of vaccines containing both BVDV I and BVDV II.

The grouping of BVDV II with BDV isolates, by phylogenetic analysis, is intriguing. The definition of the causative agent of border disease is ambiguous. There is some question whether BDV exists as a separate group within the genus Pestivirus or is a variant of BVDV adapted to growth in ovine hosts. Some groups have reported that BDV and BVDV isolates can be clearly distinguished by Mab binding (Edwards *et al.*, 1988; Shannon *et al.*, 1991; Becher *et al.*, 1994), while others using the same type of analysis have concluded that they are indistinguishable (Cay *et al.*, 1989). In comparison studies of the 5' UTR of pestiviruses, Berry *et al.* (1992) and

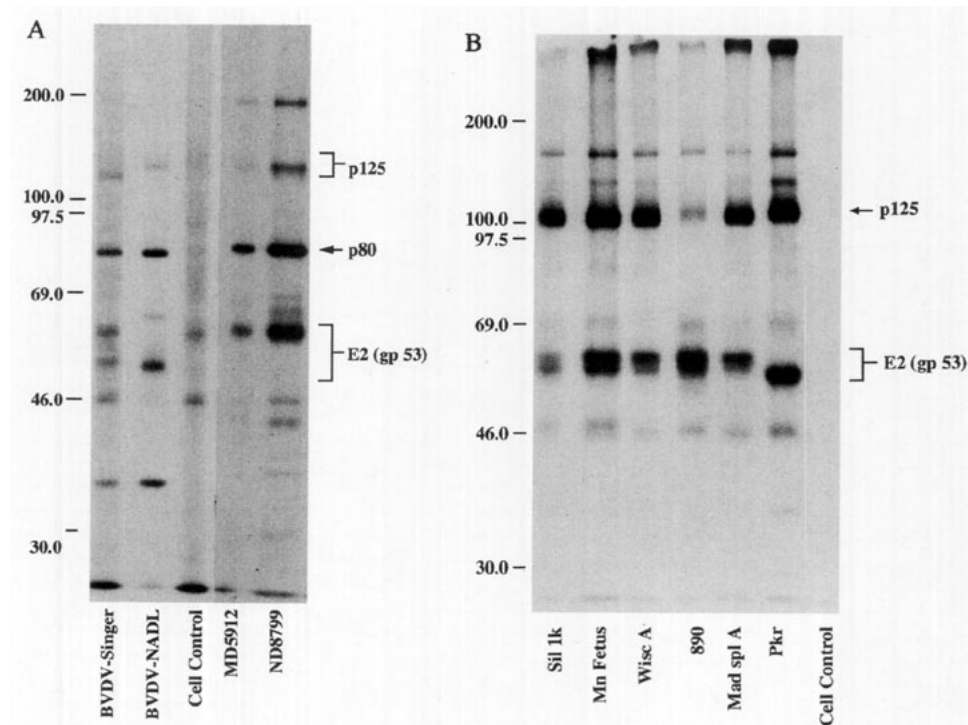


FIG. 4. Radioimmuno-precipitation of viral polypeptides from cytopathic and noncytopathic BVDV II. Examples of radiolabeled viral polypeptides produced by cytopathic (A) and noncytopathic (B) BVDV II and precipitated using polyclonal antisera prepared against BVDV are shown. The BVDV I viruses BVDV-Singer and BVDV-NADL are included on A for purposes of comparison. The identity of the band corresponding to the gp53 polypeptide was established using a Mab specific for the gp53 polypeptide. Viral polypeptides were separated on a 12.5% polyacrylamide gel with a 5% stacking gel.

De Moerloose *et al.* (1993) concluded that BVDV and BDV are distinct viruses, both equally distinct from HCV. Based on comparison of sequences coding for structural polypeptides, Becher *et al.* (1994) concluded that two types of pestiviruses may be isolated from sheep; those that are similar to BVDV and those that are more similar to HCV than BVDV but clearly distinct from both. Studies in our laboratory have shown that BVDV II isolates can be differentiated from BDV isolates by hybridization, PCR,

Mab binding, and host cell preference. Characterization of a larger number of BDV isolates will be necessary to develop a clear definition of BDV and to establish criteria by which they can be differentiated from other pestiviruses. Once the characteristics of BDV isolates are clearly defined, the practical significance of their similarity to BVDV II isolates in the 5' UTR can be assessed.

Of the 140 BVDV examined 76 (54%) were characterized as BVDV II isolates. This percentage may not be an

TABLE 3

SEGREGATION OF BVDV PCR BASED ON AMPLIFICATION OF SEQUENCES FROM THE 5'UTR AND THE REGION CODING VIRAL POLYPEPTIDE p125

BVDV I (n = 64)	BVDV II (n = 76)
Laboratory reference virus TGAC, NADL, Singer, NY-1, NEB, AUB, C24V, 7443	Fetal bovine serum 88083, 88084, 88056, 88001, 88002, 88082, 88060, 88050
Fetal bovine serum 15-260, 1942-8, 16-558, 15-735, 16-828, 16-829, 15-262, 88055, 15-265, 15-068/G2	Hemorrhagic syndrome 890, CD-87, CD-89, C-10, C-21, S5253, C-31, C-9, SL-1, SHI-3, SHI-5, SHI-9, C-8, Cali-1, C-7, C-3, C-71, C-11, C-6, C-19, C-12, C-15, C-2, C-4, 5252, SL-2, AZ-2, 793, MAD Spl A, MN fetus, Wisc A, Park
Other San, A11, 2110C, 9675, 1583-B1, Emm, Oli-HS, Bol, BurtC, SSDN, 11957, 5960, 9763, Kad, VM, ILL-NC, Oli-G7, TGAN, 1185-C, 83-7477-C, 23776/C1, 67, ED- 12, ED-26, ED-15, ED-22, ED-32, ED-6, ED-7, ED-25, ED-20, BB, Manz-C, MCT-C, NZC, 1185NC, ILLC, 190C, 826675C, WVA, 9675, SSDC, 2110NC, 639, SNC, C454, ED-24	Vaccine escapes Pur/A-11, 3590, 2541, 3659, 1831, 3237, 2251, 1826, 120C, 890962, 133
	Other ED-16, ED-17, ED-33, ED-5, ED-1, ED-3, ED-10, ED-14, ED-30, ED-4, ED-9, ED-31, ED-11, ED-18, ED-27, ED-13, ED-19, ED-23, ED-2, ED-28, 93-13628, Short, 92-8116, 277, 28508-5

accurate representation of the ratio of BVDV II to BVDV I isolates in nature. The BVDV isolates included in this survey represent reference viruses, isolations from disease outbreaks, and isolation from fetal calf serum sent to the National Animal Disease Center for testing. It has been our experience that acute, uncomplicated infections with BVDV I isolates are usually associated with mild or subclinical disease. Because we do not often receive viruses that cause mild or subclinical disease in cattle, these viruses may be underrepresented in our collection.

While a correlation between the BVDV II genotype and vaccine escape or increased virulence cannot be established by this data, it does appear that BVDV II isolates merit further study. Much of the published BVDV research, especially at the molecular level, has been done on BVDV I isolates (Collett *et al.*, 1988; Deng and Brock, 1992; Qi *et al.*, 1992; De Moerloose *et al.*, 1993). Future research in our laboratory will involve sequencing the complete genome of a BVDV II, examination of the genomes of cytopathic BVDV II isolates for insertions, and production of Mabs against BVDV II isolates.

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